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Somatic embryos of *Picea abies* behave like isolated zygotic embryos *in vitro* but with greatly reduced physiological vigour

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Use of somatic or synthetic seed is of potential interest as a micropropagation tool for superior conifer genotypes. However, whether naked or encapsulated, the most important criterion is that the asexual embryo or somatic seed must be able to simulate the zygotic embryo or true seed. This study attempted a comparison of the somatic embryo of Norway spruce (*Picea abies*) with its zygotic counterpart. A major finding was that the percentage germination of fresh or somatic embryos partially desiccated at relative humidities of 97% and 63% to moisture contents approaching those of the seed, was substantially lower. It further declined rapidly with either cold storage and/or encapsulation. This sensitivity to drying suggests that the somatic embryo/seed may behave either as an orthodox seed with limited ability to withstand desiccation or as a recalcitrant seed that cannot survive drying below a moisture content that is relatively high. As the megagametophyte contains >80% of the seed's total lipid and protein reserves, the carbon and amino acid pools available to the zygotic embryo far exceed those accessible to the somatic embryo. The conclusions are: (1) in Norway spruce the somatic seed, lacking a suitable artificial megagametophyte, does not simulate the true seed, and (2) the somatic embryo, if it behaves like an isolated zygotic embryo cultured *in vitro*, does so with greatly reduced physiological vigour.

Introduction

Many of the world's major economic crops are propagated vegetatively. It might therefore be argued that a huge potential should exist for establishing somatic embryo-based live or synthetic seed for crops such as cassava, potato, sweet potato, grapevine and sugar cane, or for species where seed production is limited or difficult to achieve because pure breeding lines cannot be established. Because of their long juvenile phases and the fact that fewer individuals can be accommodated in uniform breeding and testing sites, improvement by breeding and selection in conifers is much slower than in agronomic crops. Tree improvement is also hampered by the imprecision of early selection and the species' inability to yield inbred lines (Timmis 1998).

Kleinschmidt (1974) estimated an increase in Norway spruce of at least 10% in genetic gain from the planting of clonal propagules rather than from seed of selected families. From the mid-1970's to the mid-1980's much effort was devoted in Scandinavia, France, Germany, Canada, USA and New Zealand to the clonal propagation of conifers using rooted cuttings and micropropagation and, likewise, from the mid-1980's to date, to the application of somatic embryogenesis (Bornman 2002). However, despite initial success and promises of significant gain for the forestry industry, for example via mass propagation of superior genotypes (Gupta *et al*. 1991), commercialisation of neither micropropagation- nor somatic embryo-based technology has occurred to any significant degree.

In the current study we posed two questions concerning Norway spruce: does the somatic seed, either naked or encapsulated, behave like the true seed? Were the answer to be no, then the ensuing question is whether the somatic embryo behaves like a zygotic embryo *in situ* or like an isolated zygotic embryo *in vitro*? The latter question was prompted by the work of Faure *et al*. (1998) who found that precociously germinating somatic embryos of grapevine had lower levels of IAA and ABA than their germinating zygotic counterparts. This suggested to these authors that the absence of an ABA peak was preventing the switch from the mid- into the late-embryogenesis phase.

It is during the phase of late-embryogenesis that an orthodox seed becomes desiccated and enters into a dehydrated resting or quiescent phase, separating the events of embryogenesis *per se* from those of germination (Thomas 1993). Embryogenic tissue in culture undergoes continuous growth (Gray and Compton 1993) which, if not arrested artificially, results in precocious germination and prevents the

development of normal plants. The conifer has proved to be no exception. According to Galau *et al*. (1991) precocious germination occurs when, in the absence of late-embryogenesis, there is no separation between mid-embryogenesis and germination.

If synthetic or somatic seed that derived from embryogenic tissue of the true seed were to simulate zygotic seed, then the former has to be artificially induced to mimic as closely as possible the events that occur in embryo development in the latter. We thought that some comparisons of the conifer somatic embryo with its isolated zygotic counterpart might shed some light on this.

Materials and Methods

Plant material

Standard procedures (Becwar *et al*. 1990, Klimaszewska 1989, Klimaszewska and Smith 1997) were used to initiate and maintain an embryogenic cell line from immature somatic embryos of a control-pollinated *Picea abies* tree. The initiation medium contained 30mM sucrose, 10µM 2,4-D and 5µM BA, and the maturation medium 80mM sucrose and 25µM ABA. Before transfer to maturation medium, embryos were kept on maturation medium minus ABA for one week to remove possible residual effects of the auxin and cytokinin. Seed from the same source was used for comparative analysis. Seeds were soaked for 10min before dissecting the embryo from its megagametophyte. Only well-formed somatic embryos of similar phenotype, presumed to resemble the zygotic embryos in morphology, were picked from the embryogenic cultures after 6–8 weeks. The tissues were then placed in 1.5ml microfuge tubes, frozen in liquid nitrogen and stored at -70°C until used for chemical analysis. A portion of the seed was placed on moist filter paper and imbibed in the dark at 26 ± 2°C for five days. Embryos and megagametophytes from the 5-day-imbibed seed were also dissected and stored as above.

Quantification of triacylglycerol (TAG), protein, sucrose, D-glucose and D-fructose

Triacylglycerols were extracted using a modification of the method of Feirer *et al*. (1989). Tissues with a fresh mass of 5–60mg were homogenised with a pestle fitted to a 1.5ml microfuge tube containing 100µl iso-propanol. The volume was adjusted to 1ml with iso-propanol and the tubes shaken for 15min before centrifuging at 20 000g for 5 min. Then, 800µl of supernatant were added to test tubes containing 0.8g of alumina (activity grade 1, type WN-3, Sigma) and 1.8ml iso-propanol. The samples were mixed gently for 15min before centrifuging at 1 500g for 5min to sediment the alumina. TAG quantification was carried out as follows: the 800µl aliquots of supernatant were incubated at 60°C with 1M KOH for 5min and allowed to cool to room temperature before addition of 200µl of a sodium periodate solution (12mM NaIO4, 2M acetic acid). After 10min, 1.2ml colour reagent (40ml 2M ammonium acetate, 150µl acetylacetone, 80ml isopropanol) were added and the samples placed in a water bath for 30min. The absorbance of the samples was measured at 410nm and the concentrations of TAG determined using triolein (C18:1,-CIS-9, Sigma) as standard.

Soluble protein fractions were determined according to Kruger *et al*. (1983). Briefly, samples of 5–60mg fresh tissue mass were placed in 1.5ml microfuge tubes with 100µl extraction buffer (100mM Tris-acetate pH 8, 2mM MgCl2, 1mM PMSF, 10% glycerol and 2% insoluble PVP) and homogenised with a glass pestle. A volume of 900µl extraction buffer was then added and the samples shaken briefly before being left to stand for 30min at room temperature. The samples were then centrifuged at 10 000g for 5min and the supernatants assayed for buffer-soluble proteins. The pellet containing the buffer-insoluble protein was re-extracted in 1ml of Laemmli buffer (Laemmli 1970). The samples were boiled for 15min and then centrifuged at 10 000g for 20min. Both soluble and insoluble protein fractions were assayed using the Bio-Rad™ protein microassay procedure, based on the method of Bradford (1976) using bovine serum albumin as standard.

Quantification of sucrose, D-glucose and D-fructose was by a modified method of Stitt *et al*. (1983). Tissue (15–200mg fresh mass) was freeze-clamped in liquid nitrogen and powdered using a pestle fitted to a 2ml microfuge tube. The carbohydrates were extracted for 15min at room temperature in 1.2ml 20mM HEPES pH 8.5 plus 2mM EDTA, 0.8ml methanol, 0.3ml chloroform and 0.7ml H_2O. The samples were centrifuged at 10 000g for 5min and the aqueous supernatant used for the carbohydrate analysis. Sucrose, D-glucose and D-fructose were quantified using a Boehringer-Mannheim kit (Roche Diagnostics).

Histology

Light microscopy

For light microscopy tissues were fixed in 6% glutaraldehyde in 0.15M phosphate buffer pH 7.4 and embedded in glycolmethacrylate (O'Brien and McCully 1981). Sections (c. 2–4µm) were cut with glass knives and stained for protein with toluidine blue and mercuric bromphenol blue (Pearse 1961).

Electron microscopy

For transmission electron microscopy (TEM) material was fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer at pH 7.4 for 4h. This was followed by 3 x 10min buffer rinses (0.1M; pH 7.4). Post-fixation was done in 1% aqueous OsO_4 for 2h and dehydration in an ethanol series of 30%, 50%, 70%, 90% and three changes of 100% each for 15min. Infiltration and embedding was done with a modified Quetol 651-epoxy resin (Van der Merwe and Coetzee 1992). Thin sections were contrasted with uranyl acetate (10min) and lead citrate and viewed and photographed in a Philips EM 301 operated at 60kV.

For scanning electron microscopy (SEM) the method of Barnes and Blackmore (1984) was followed. Briefly, this entailed fixation in 1% OsO_4 in 0.1M sodium cacodylate buffer (pH 7.4) for 2h at room temperature. The fixed tissue was rinsed in the same buffer (3 x 10min) and treated with 15%, 30% and 50% aqueous dimethyl sulphoxide (DMSO) for 30min in each solution. Thereafter, the tissue pieces were frozen in liquid nitrogen and freeze-fractured with a

razor blade. Fragments were allowed to thaw in 50% DMSO, rinsed in buffer, and treated with 0.1% aqueous OsO_4 for seven days at 4°C. Following three buffer rinses, the tissue was dehydrated through a graded series of ethanol as used for TEM, and then critical point dried. Tissue pieces were mounted on stubs and exposed to RuO_4 (from a 0.5% solution of RuO_4) vapour for 30min to enhance conductivity (Van der Merwe and Peacock 1999). Material was studied using a JEOL 6000F field emission scanning electron microscope.

Partial desiccation and germination

Partial desiccation of somatic embryos was based on the work of Bomal and Tremblay (1999). It involved drying for 72h in the dark at 26 ± 2°C in a 7.5 litre desiccator, but at only two levels of relative humidity (RH), namely 63 ± 2% and 97 ± 2%, respectively, generated from saturated solutions of NH_4NO_3 and Na_4HPO_3. Twenty-five embryos (FW c. 30mg) were transferred to small (35mm-diameter) petri dishes. Eight such dishes (total of 200 embryos) were positioned inside a large (145mm diameter) petri dish that was placed covered but unsealed in the desiccator. Relative humidity in the desiccator was verified hygrometrically. Freshly harvested somatic embryos served as control.

To determine the effects of desiccation and artificial encapsulation on germination, seed, isolated zygotic embryos and freshly harvested somatic embryos were germinated directly, while the partially dehydrated somatic embryos were first re-hydrated following the procedure of Anandarajah and McKersie (1990). Surface-sterilised seed was germinated in standard germination trays at 26 ± 2°C. Somatic embryos and freshly-isolated zygotic embryos were placed either in baby food jars on 3.5g l^{-1} Gelrite™ slopes containing the basal salts of the Schenk and Hildebrandt (1972) medium at one-quarter strength, 60mM sucrose, 0.1M mannitol and 5g l^{-1} activated charcoal or encapsulated and introduced into liquid culture (see below). Germination of seeds and isolated zygotic embryos, kept at 26 ± 2°C in darkness, was scored after seven and 21 days and of the fresh and partially desiccated embryos after 21 days. Criteria for germination were a protruding and elongating radicle and expanding cotyledons. Conversion of the germinating embryos to plantlets was not undertaken. To determine the response to cold storage, a set of similarly treated tissues was kept at 4 ± 1°C for 30 days and 90 days. Alternatively, a batch of fresh somatic embryos was cold-treated for the same period before dehydration, encapsulation and germination of the respective embryos, as above.

Synthetic seed

Isolated zygotic and partially desiccated somatic embryos of approximately equal dimensions were selected. Mean lengths of zygotic and somatic embryos were 3.3 ± 0.3mm and 3.3 ± 0.5mm and diameters at the midpoint of the hypocotyl-root axes 0.8 ± 0.1mm and 1.0 ± 0.2mm, respectively. The respective mean fresh and dry masses (mg) were 6.6 ± 0.7 and 0.4 ± 0.1 for zygotic and 8.3 ± 0.7 and 0.3 ± 0.1 for somatic embryos. A 2% (w/v) solution of Na-alginate (Redenbaugh *et al*. 1986) containing 5g l^{-1} activated charcoal was prepared in which somatic and isolated zygotic embryos were suspended. The suspended embryos were dropped into 200ml 50mM $CaCl_2$ by means of a plastic bulb pipette with an internal nozzle diameter of 4mm. The resulting Ca-alginate capsule was allowed to stabilise for 30min, providing a gel hardness of about 750mg/bead. The mean diameter of the alginate beads was 5.5 ± 0.5mm.

The synthetic seeds were transferred to simple but functional mini-bioreactors constructed from Magenta™ tissue culture containers. The culture medium consisted of one-quarter strength Schenk and Hildebrandt (1972) macro- and micronutrients, as for germination, but minus gelling agent and activated charcoal. Also omitted was the iron-EDTA source, as a precaution against possible chelation of calcium and subsequent liquefaction of the Ca-alginate gel. Fifty encapsulated embryos were introduced into 50ml filter-sterilised medium in the autoclaved bioreactors and the assembly attached to an air pump. Air was admitted and vented through 0.45µm filters.

Results

Nutrient reserves

A Norway spruce seed contains on average 400µg total protein, 80% of which is located in the megagametophyte (Figure 1). On average, a somatic embryo of approximately similar fresh mass contains one-quarter of this amount. After five days imbibition, total protein in the megagametophyte had decreased by 30%, while that of its embryo increased by about 20%. Somatic and zygotic embryos contained the same levels of soluble protein (30–40µg/embryo, data not presented) but the former had significantly higher amounts of insoluble protein (c. 65 as compared with 45µg/embryo). The seed contains about 700µg of TAG, of which >80% is localised in the megagametophyte. Despite its relatively low lipid content, the zygotic embryo still has about 70% more TAG than the somatic embryo. By day five of imbibition TAG levels had dropped by 18% in the megagametophyte and 33% in the zygotic embryo. On average, somatic embryos contained significantly less sucrose than zygotic embryos, about 20 as compared with 32µg/embryo. D-glucose and D-fructose were below detectable levels in the zygotic embryo and megagametophyte of unimbibed seed, as well as in the somatic embryos. However, after day five of imbibition, levels of glucose and fructose had increased to detectable levels in the zygotic embryo and megagametophyte. Expressed as a percentage of dry mass, the five-day imbibed zygotic embryo contained about 1.9% and 1.3% glucose and fructose, respectively, levels that were about five times higher than those in the imbibed megagametophyte (data not shown).

Morphology and anatomy

Figures 2A and 2B show the distribution of protein reserves in the megagametophyte-zygotic embryo complex at the stage of early germination. Mobilisation of the protein reserves in the megagametophyte occurs in a centrifugal direction, from the inner cells abutting on the corrosion corridor (CC) in the direction of the outer epidermis. Cells and

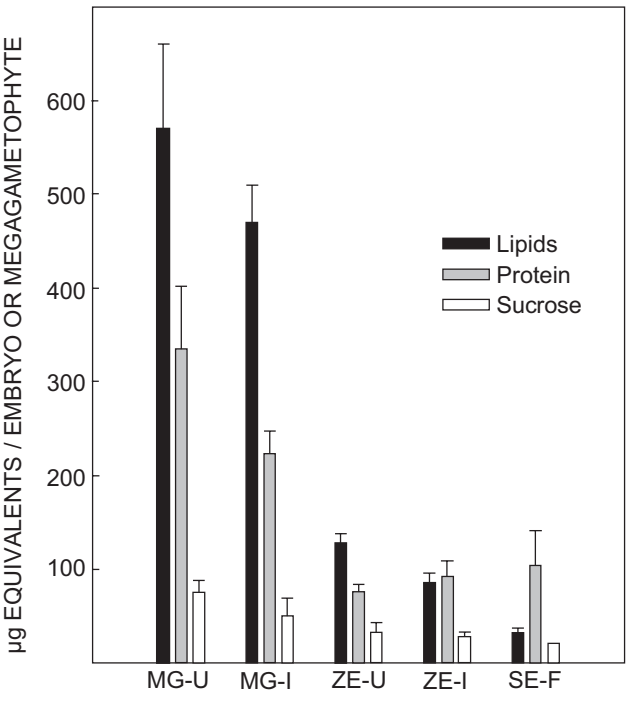

Figure 1: Quantitative differences in lipids (triacylglycerols as triolein equivalents), total protein and sucrose (as glucose equivalents) among freshly-harvested somatic embryos (SE), and unimbibed and five-day imbibed zygotic embryos (ZE, ZE-I) and megagametophytes (MG, MG-I). Although designated *fresh*, the somatic embryos had presumably undergone partial desiccation while on maturation medium (moisture content 65% after six weeks of maturation). Each datum point is the mean of three independent determinations ±SE

protein bodies of the megagametophyte are larger than those of the embryo. In addition to protein bodies (PB), most cells contain a single eccentric protein globoid (G), characteristic of gymnosperm cells, but some contain two. Staining with bromphenol blue (Figure 2C) confirms the presence of protein in organelles that are densely packed in the megagametophyte's storage cells. Lipid reserves are not seen as they were extracted during dehydration. Protein body vacuoles in the cells of the zygotic embryo (Figure 2D, PV) indicate that at this stage of germination mobilisation of embryo protein reserves had already occurred. In fact, protein reserves in the embryo are depleted rapidly and protein bodies form vacuoles after one day of imbibition. At the approximate midpoint of the hypocotyl, cells in the cortex of the zygotic embryo (Figure 2D) are compactly arranged, whereas those of the somatic embryo's cortex (Figure 2E) are more loosely arranged, displaying larger intercellular spaces. Transverse sections midway through the cotyledons show a developing vascular bundle in the zygotic embryo (Figure 2F, arrow) as compared with procambial strands (Figure 2G, arrow) as well as less compact mesophyll in the somatic embryo.

The densely packed cytoplasmic nature of the cells of the zygotic embryo is confirmed at the fine structural level (Figures 3A and 3B). At the stage of early germination, evidence of the cellular activity responsible for mobilisation of nutrient reserves in the embryo and its adjacent megagametophyte is reflected in well-developed dictyosomes (D), mitochondria (M) and glyoxysomes (G). Cells of the megametophyte (Figure 3C–F) contain protein bodies (PB) surrounded by numerous lipid or oil bodies (LB). To enhance resin infiltration and staining of lipids in the megametophyte, the tissue was treated with 1% OsO_4 in acetone. The section represented by F, which shows depleted protein bodies (or vacuoles, PV) encircled by lipid bodies, was not contrasted with uranylacetate or lead, confirming that the contents of the encircling organelles are indeed lipids. Proteins appear to be hydrolysed before lipids, as seen when comparing an earlier (E) with a later (F) stage.

Figure 4 shows different aspects of somatic embryo development. In Norway spruce, embryogenic tissue can be initiated from either immature or mature zygotic embryos isolated from the seed (A). Under appropriate conditions an embryo-suspensor mass (ESM) may be formed that consists of interspersed proembryonic and non-embryogenic (callus) tissue. Frequent subculture of the ESM helps to maintain embryogenicity. Figure 4B depicts a somatic embryo at the globular stage, surrounded by embryogenic tissue and callus (arrow). For the cell line used, we estimated a yield of about 250 somatic embryos/(g FW) of ESM (Figures 4C and 4D), of which c. 25%, visually judged as normal, were selected for the experiments. In comparison with the zygotic embryo (E), the phenotype of the somatic embryo can vary greatly, from normal (F, G) to malformed or teratogenic (H) morphotypes.

Partial desiccation and germination

The effects of drying at two relative humidities and of cold storage on the germination of either naked or alginate-encapsulated somatic embryos are shown in Table 1. Intact seed and isolated zygotic embryos served as controls. The latter maintained a high frequency of germination except for encapsulated zygotic embryos that had been in cold storage for both one and three months. Fresh somatic embryos displayed a minimum-*mean*-maximum variation in moisture content of 49–*57*–69%. Drying at RH97 reduced both the range in variation and the moisture content of the embryos to 17–*23*–31, while treatment at RH63 caused a further reduction (6.3–*7.4*–8.3), resulting in moisture contents of the somatic embryos comparable to those of the seed and isolated zygotic embryos. Cold storage had a deleterious effect on the somatic embryo germination.

Excised and encapsulated zygotic embryos showed high frequencies of germination (Table 1), with their radicles emerging from the beads by day seven. By day 21, emerging shoots had cracked apart the gel capsules. Encapsulated somatic embryos responded slowly. Figure 4 shows somatic embryos newly encapsulated in alginate beads (I) and after 21 days in an aerated liquid culture (J). Compared with zygotic embryos, few radicles of somatic embryos emerged from the beads, in contrast to hypocotyls and cotyledons, which often were distended (Figure 4J). Generally, encapsulation affected germination negatively, especially that of somatic embryos held in cold storage for a month or longer.

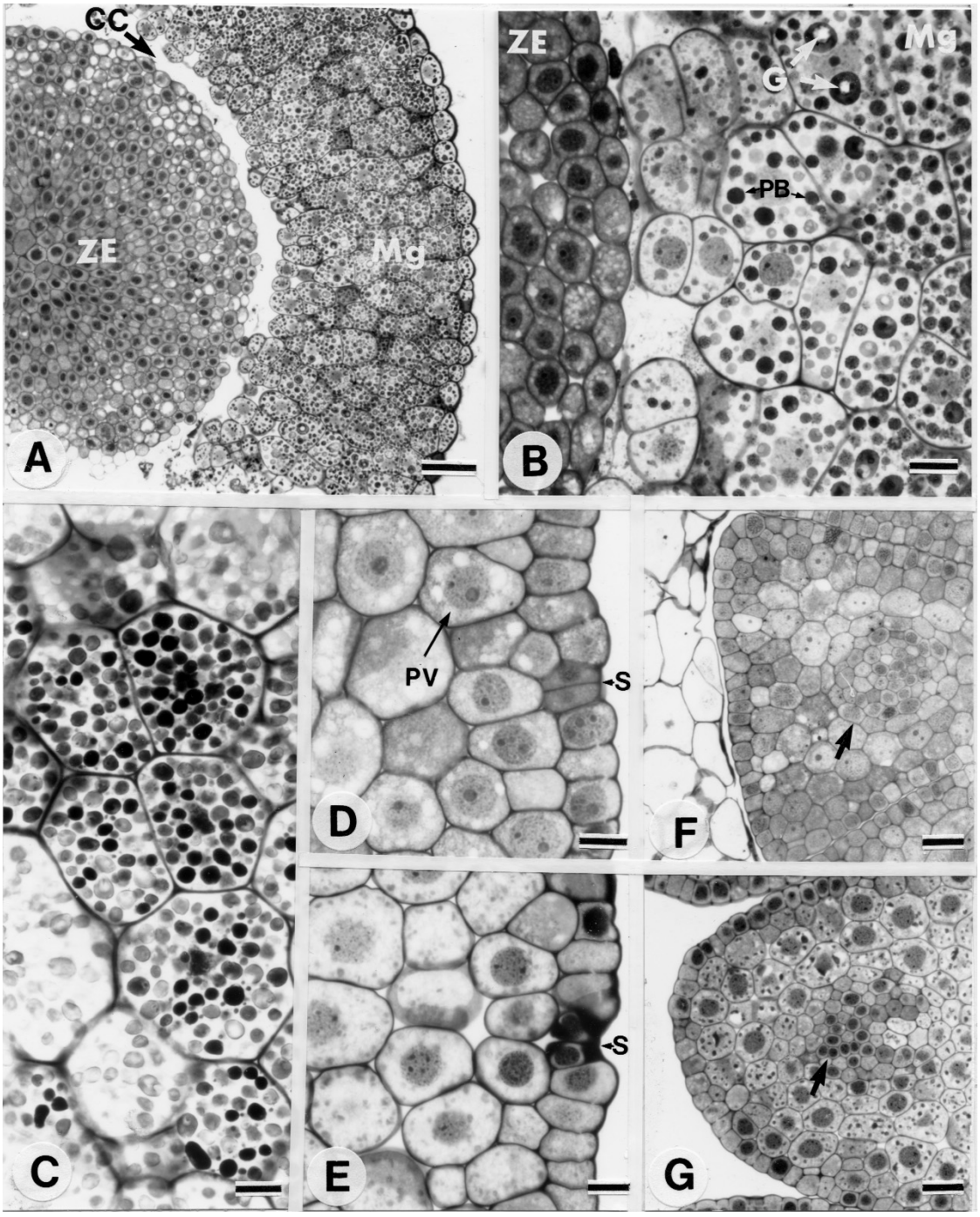

Figure 2: Transverse sections of glycol methacrylate-embedded megagametophyte, zygotic embryo and somatic embryo tissues of Norway spruce (*Picea abies*) at the early germination stage (except for section C, which represents unimbibed tissue). Sections A and B and D–G were stained with toluidine blue and section C with bromphenol blue. (**A**) Overview of part of the seed showing the zygotic embryo separated by a corrosion corridor from the surrounding megagametophyte. (**B**) Higher magnification of part of the megagametophyte-embryo complex. Mobilisation of protein reserves in the megagametophyte occurs in a centrifugal direction starting from the inner cells adjacent to the corrosion corridor. Most protein bodies contain single globoids (G) but some contain two. Protein body vacuoles in cells of the zygotic embryo indicate that mobilisation of protein reserves had already occurred. Many of these vacuoles will later fuse. (**C**) Staining with bromphenol blue confirms the presence of protein in protein bodies of the megagametophyte cells. (**D**, **E**) At the midpoint of the hypocotyl, cortex cells of the zygotic embryo (D) are compact and display numerous protein body vacuoles, whereas cells of the cortex of the somatic embryo are more loosely arranged, with larger intercellular spaces. (**F**, **G**) Sections midway through the cotyledons show a developed vascular bundle in the zygotic embryo (F, arrow) and a procambial strand (arrow) as well as a more loosely arranged mesophyll in the somatic embryo (G).
CC, corrosion corridor; G, globoid; Mg, megagametophyte; PB, protein body; PV, protein body vacuole; S, stoma; SE, somatic embryo; ZE, zygotic embryo. Bars represent 50µm in A, 10µm in B–E and 20µm in F and G

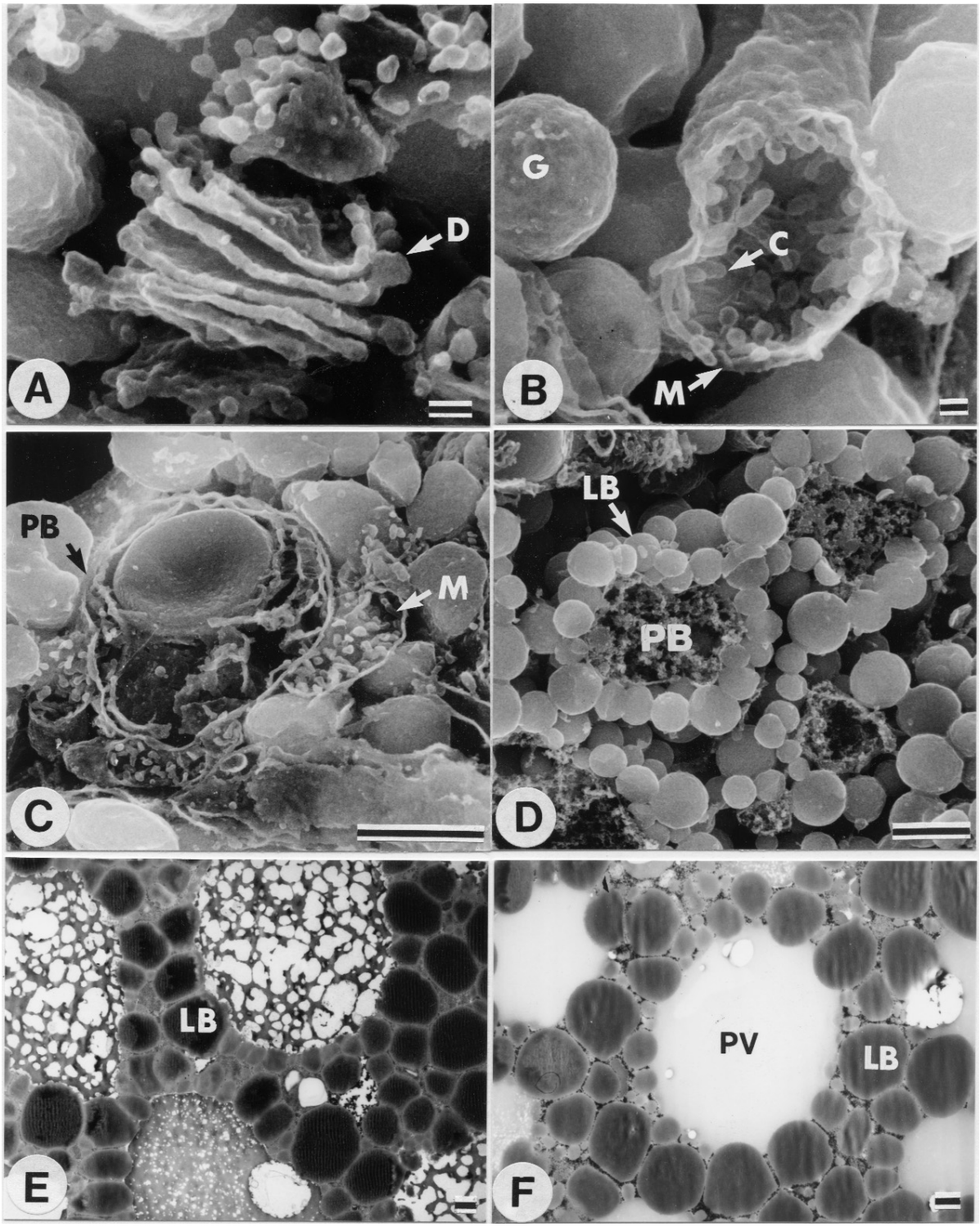

Figure 3: Scanning (A–D) and transmission (E–F) electron micrographs showing details of zygotic embryo (A, B) and megagametophyte (C–F) cells at the early germination stage.(**A**) Early intracellular activity in embryo cells, responsible for mobilisation of nutrient reserves in the megagametophyte, is evidenced by well-developed dictyosomes and mitochondria. (**B**) The fractured mitochondrion reveals cristae formed by the internal membrane. Glyoxysomes (G) are present. (**C**, **D**, **E**, **F**) Cells of the megagametophyte contain protein bodies surrounded by numerous lipid or oil bodies. To ensure staining of lipids by OsO_4, the tissue represented by F, which shows depleted protein bodies or protein body vacuoles encircled by lipid droplets, was not contrasted with uranylacetate or lead. Proteins appear to be hydrolysed before lipids, as seen by comparing an earlier (E) with a later (F) stage of mobilisation.
C, cristae; D, dictyosome; G, glyoxysome; L, lipid body; M, mitochondrion; PB; protein body; PV, protein body vacuole. Bars represent 100nm in A and B and 1µm in C–F

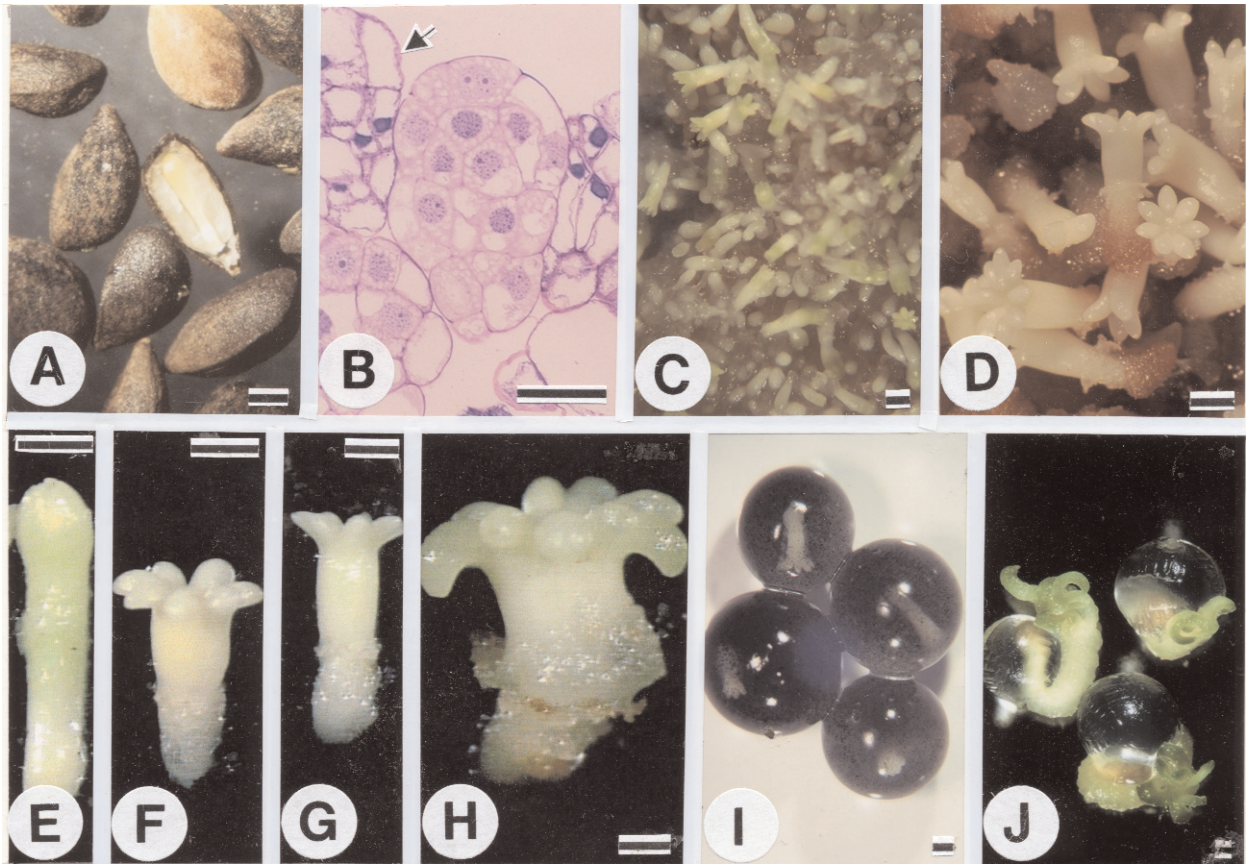

Figure 4: Montage demonstrating different aspects of zygotic and somatic embryo development in *Picea abies*. (**A**) Section through a 10-minute-imbibed seed showing the embryo embedded in the megagametophyte. (**B**) Globular stage somatic embryo surrounded by embryogenic tissue and callus (arrow). (**C**, **D**) Somatic embryos at the torpedo stage, each displaying six or more cotyledons. (**E**) A zygotic embryo isolated from a one-day-imbibed seed, has already commenced elongation of its hypocotyl-root axis. (**F**, **G**, **H**) Mature (F), mature and partially desiccated (G) and teratogenic (H) somatic embryos. (**I**, **J**) Somatic embryos encapsulated in alginate (I) and with distorted shoots emerging from the bead after 21 days in an aerated liquid culture (J). Except for B (50µm), all bars represent 1mm

Discussion

Nutrient reserves and histology

Lipids and proteins are the major reserves in gymnosperm seeds, where the largest proportion is localised in the megagametophyte (Butler *et al*. 1979, Stone and Gifford 1997, 1999). Germination triggers the hydrolysis of the triglycerides that are stored in oil bodies, especially abundant in the megagametophyte, to free fatty acids that are subsequently oxidised to produce acetyl CoA. The acetyl CoA is metabolised and the succinate that is produced transported from the glyoxysome to the mitochondrion, where it is converted to oxalacetate. In the cytoplasm, oxalacetate is then converted to glucose via gluconeogenesis and then to sucrose. This mobile form of carbon, together with the amino acid breakdown products of the protein reserves, is available to the germinating embryo.

In the zygotic embryo of Norway spruce, the TAGs are present at 3.5 times the levels of those found in morphologically equivalent somatic embryos. After a five-day period of imbibition, the levels of TAG in the zygotic embryo had dropped significantly but were still higher as compared with the somatic embryos (Figure 1). The zygotic embryos contained less insoluble protein than its equivalent somatic embryo but there were no significant differences in the levels of soluble proteins. Compared with its counterpart *in situ,* an isolated zygotic embryo accumulates carbohydrates to a lesser extent when cultured *in vitro* (Stone and Gifford 1999).

An earlier study (Bornman *et al*. 2001) reported no significant differences in cell number per unit tissue volume between freshly harvested zygotic and somatic embryos, and concluded that the larger tissue volume often displayed by the somatic embryo was reflected in a larger per cell volume. As zygotic and somatic embryos of approximately similar dimensions were selected, neither cell number nor tissue volume was determined in this study. However, the visual impression at the level of the light microscope that the cells of the zygotic embryo are cytoplasmically more dense and contain a greater proportion of nutrient reserves than those of the somatic embryo, was borne out at the fine structural level and by biochemical analysis.

Table 1: Effects of desiccation and cold storage on percentage germination of naked (N) or encapsulated (E) Norway spruce somatic embryos (SE) as compared with seed and isolated zygotic embryos (ZE). Embryos were dried at relative humidities of 97% and 63%. Percentage germination is the mean of 50 embryos per treatment (treatments repeated three times) showing elongation of the hypocotyl-root axis and cotyledon expansion

Treatment	Moisture content, % FW	Germination (%)			
		after		21 days after storage at 4 ± 1°C for	
		7 days	21 days	1 month	3 months
Seed	6.8	92	95	93	93
ZE-N	7.2	78	89	55	43
ZE-E	7.2	57	73	14	10
SE-F,N	57.0	-	66	16	2
SE-F,E	57.0	-	35	4	0
SE97-N	19.3	-	77	23	4
SE97-E	19.3	-	41	7	0
SE63-N	7.4	-	21	0	0
SE63-E	7.4	-	13	0	0

Partial desiccation

Compared with fresh embryos, partial desiccation of somatic embryos for 72h at RH97 and 63% resulted in lower and less variable moisture contents. The mean percentage germination, considered as elongation of the hypocotyl-root axis and expanding cotyledons, was similar for the freshly-matured and RH97-exposed somatic embryos, an observation in accord with the results of Bomal and Tremblay (1999), although the percentages in our case were considerably lower. Desiccation at RH63 resulted in embryo moisture contents comparable with those of the isolated zygotic embryos but with incomparably poorer germination. Storage at 4°C for one or three months affected germination of somatic embryos negatively, with those exposed to RH63 unable to survive.

Whereas the literature on conifer somatic embryogenesis is substantial, relatively fewer and often confusing data are available on the effects or benefits of partial desiccation on germination and plantlet development. The confusion probably arises from the use of non-standardised procedures. Drying times and drying temperature, the embryo mass used and the ratio of embryo mass to desiccator volume (Bomal and Tremblay 1999) are often at variance. Lack of precise RH measurements and large discrepancies between germination percentages and frequencies of conversion of the germinating propagules to plantlets are additional factors that make it difficult to compare results from different laboratories.

Senaratna *et al*. (1989) and Tetteroo *et al*. (1995) showed that slow drying was essential for the acquisition by somatic embryos of desiccation tolerance in lucerne and carrot, respectively. This has also been established for interior spruce (Roberts *et al*. 1990), sitka spruce (Roberts *et al*. 1991), white spruce (Attree *et al*. 1991), black spruce (Beardmore and Charest 1995) and hybrid larch (Lelu *et al*. 1995, Dronne *et al*. 1997). It is assumed that partial desiccation of somatic embryos improves germination and enhances plantlet recovery. However, in black spruce, Bomal and Tremblay (1999) found that a fast drying rate to 4–5% of embryo moisture content obtained under 63% RH was detrimental to germination and plant development, when compared with relative humidities of 79–97% that generated embryo moisture contents of 7–19%. They found that fresh embryos and those dried at 97%, 88% and 79% RH germinated rapidly and at a high percentage (>90%). However, conversion to plantlets at RH97 was only 40%, but nevertheless still twice as effective as no drying or drying at 88% RH. A relative humidity treatment at 79% yielded 7% plantlets; that at 63%, zero. Gorbatenko and Hakman (2001) working with somatic embryos of Norway spruce produced in liquid culture, achieved 92%, 72%, and 15% germination of fresh, RH90 and RH81 drying treatments, respectively, with no germination at RH63 and RH31. However, if passed through atmospheres of successively reduced RH (from 90–31%) over a period of three weeks, the embryos showed a high level of desiccation tolerance (95% survival) and development into plantlets (40%).

In spite of different experimental approaches, neither Bomal and Tremblay (1999) nor Gorbatenko and Hakman (2001) were able to establish normal plantlets of black spruce or Norway spruce from somatic embryos subjected to desiccation at RH63. In contrast to the steep decline in embryo survival with progressively decreasing relative humidity from 90% to 31%, Gorbatenko and Hakman (2001) reported 100% germination of fresh (i.e. non-artificially-dried) embryos and a plantlet recovery frequency of 92%. In black spruce (Bomal and Tremblay 1999), non-dried embryos germinated to 100% but with a conversion frequency of only 27%.

The ultimate criterion of germination has to be the frequency at which plantlets can be produced. During maturation the somatic embryo enters from a phase of disorganised, continuous growth into a quiescent phase in response first to a rise in ABA and then to desiccation. If embryo germination is defined as the resumption of growth, in terms of root elongation and expanding cotyledons, yet failing to allow plantlet development, it may be as a result of partial dormancy caused perhaps by the presence of ABA. Dronne *et al*. (1997) found that the ABA content in hybrid larch somatic embryos fell significantly after seven days desiccation at 98% RH at 4°C, but was still present at c. 50pmol /embryo.

Somatic seed

Kitto and Janick (1985) initiated artificial coating of asexual propagules, a technology that became of interest as somatic embryo systems in carrot, celery and lucerne were already successfully established (Redenbaugh *et al*. 1986, Florin *et al*. 1993). The most important criterion for its usefulness is that the synthetic seed pellet should allow germination of embryo-forming plants at frequencies comparable to normal orthodox seed. To be truly functional, somatic seed must mimic true seed in aspects of quiescence, storage ability and vigour (McKersie and Bowley 1993). In agreement with results on carrot (Li 1993) and *Pinus patula* (Sparg *et al*. 2002) synthetic seed, the somatic embryos of Norway spruce failed to germinate if the gel capsule lacked activated charcoal. As in the case of *P. patula***,** longevity of the encapsulated somatic embryos, especially those dried at RH63, decreased rapidly after cold storage. This was also true of encapsulated zygotic embryos but to a lesser degree. Encapsulated zygotic embryos displayed greater germinative vigour.

After extensive testing, Redenbaugh *et al*. (1986) concluded that Na-alginate was the most suitable encapsulating matrix. A number of variations to the encapsulation process have since been attempted in order to improve efficiency but as far as we know none have been able to mimic the natural seed's nutrient composition. In the conifer, provision for an artificial megagametophyte for the somatic embryo remains a laboratory exercise.

As somatic embryos, especially those exposed to drying at low relative humidity, can be dried down to moisture contents equivalent to or lower than those of the seed and isolated zygotic embryos, but yet fail to develop into plants, it is possible that they are highly sensitive to desiccation. Cold storage and encapsulation are additional factors that serve only to aggravate the germination response. It might be that in contrast to the true (orthodox) seed the somatic seed in spruce behaves either as an orthodox seed with limited desiccation ability or as a recalcitrant seed that cannot survive drying below relatively high moisture contents and rapidly loses viability. Were this to be the case, it will require different techniques such as flash drying, fast freezing and cryostorage for maintaining and extending longevity of the artificial seed (for a review of seed recalcitrance see Berjak and Pammenter, 2001). As the megagametophyte contains more than 80% of the seed's lipid and protein reserves, the total carbon and amino acid pools accessible to the zygotic embryo far exceed those available to the somatic embryo. In Norway spruce and perhaps also other conifers, the somatic seed does not emulate the true seed, probably because it has thus far not been possible to provide the asexual embryo with a suitable artificial megagametophyte. The somatic embryo behaves more like an isolated zygotic embryo *in vitro* but with greatly reduced physiological vigour.

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